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Molecular and functional analysis of the poly-β-hydroxybutyrate biosynthesis operon of *Pseudomonas* sp BJ-1

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ABSTRACT. The operon comprising the genes for poly-β-hydroxybutyrate (PHB) biosynthesis in *Pseudomonas* sp BJ-1 was cloned and sequenced. Sequence analysis of 8991 bp revealed that the regions contain two related operons. The first operon contains the three genes *phbA*, *phbB* and *phbC*, and the other contains the two genes *flp1* and *flp2*. The deduced amino acid sequences of PHBA and PHBB showed high identity with other bacterial PHB genes. Transcription of the three genes of the first operon is controlled by a single hypothetical promoter region, whereas the other two *flp* genes are controlled by two hypothetical promoter regions. Analysis of expressed protein at different times showed that PHBA protein levels increased from 0 to 4 h; PHBB and PHBC showed similar kinetics. Detection of enzyme activity showed three proteins with bioactivity and biological function in the synthesis of PHB intermediates.

Key words: Poly-β-hydroxybutyrate; Biosynthesis; Operon; *Pseudomonas* sp BJ-1

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INTRODUCTION

Plastic chemicals can hardly be decomposed in nature. They are harmful to human health and the environment (Kaneko et al., 2006). Now, people have become more concerned about protecting the environment. To reduce white pollution, degradable plastic is an inspiring measure to solve the problem, and more scientists are engaged in related research (Agamuthu and Faizura, 2005). Polyhydroxyalkanoates (PHA) are a kind of water-insoluble biodegradable storage polymers (Yan et al., 2006). The most common PHA is poly-β-hydroxybutyrate (PHB), and this polymer can accumulate up to 90% of the cellular dry weight of some bacteria (Uchino et al., 2008). PHB as a kind of PHA is a store of polyester in microorganisms and similar to stores of starch or fat in plants or animals. PHB have received increased attention because of their thermoplastic or elastomeric properties, which resemble those of petroleum-based plastics, yet they are completely biodegradable (Chien et al., 2007).

There are many natural bacteria or even plants that can directly produce PHB (Nikel et al., 2006). PHB biosynthesis genes including *phbA* (encoding 3-ketothiolase), *phbB* (encoding acetoacetyl-CoA reductase), *phbC* (encoding PHB synthase), *phaP*, *phaR*, and *phaQ* have been cloned from *Pseudomonas*, *Alcaligenes*, *Streptomyces aureofaciens*, *Azospirillum brasilense*, *Rhizobium meliloti*, *Synechocystis* sp, *Rhodobacter sphaeroides*, *Rhodospirillum rubrum* (Seo et al., 2003; Lee et al., 2004; Trainer and Charles, 2006; Uchino and Saito, 2006; Wang et al., 2006). Analyses of the PHB operon in many bacteria show that the *phbA* and *phbB* genes are relatively conserved. Three genes are located in an operon and regulated by identical promoters (Kichise et al., 1999; Matsusaki et al., 2000).

We screened the *Pseudomonas* sp BJ-1 strain from soil, which possesses high PHB production, and the BJ-1 strain PHB operon was cloned and sequenced. DNA and amino acid sequence alignment confirmed the PHB biosynthesis gene structure and composition. The operon gene array is different from that of other bacteria as well as *Pseudomonas* sp. PHB was synthesized using acetyl CoA substrates and catalyzed by three protein, which confirms that the PHB operon shows high diversity in different strains.

MATERIAL AND METHODS

Strain and plasmid

Pseudomonas sp BJ-1 strain was used in this study. *Escherichia coli* DH5α strain was grown on LB medium at 37°C. The expression plasmid pBV220 was used for gene function validation. *E. coli* TG1 (Invitrogen) was used as the host for expression plasmid propagation. Ampicillin (100 µg/mL) was added according to the presence of plasmid-borne resistance genes.

PCR amplification and DNA sequencing

Polymerase chain reaction (PCR) primers were designed based on conserved sites in the PHB operon. The PCR products were cloned into a PGEM-T Easy Vector (Promega). The primers for the PHB operon consisted of forward: CCGGGCCCGCCTTCGCAGGCT and reverse: GGTGTCGCCGAGCAGGATCGA. The vector was sequenced by Applied Biosystems 3730 DNA sequencer at TaKaRa Biotechnology Co., Ltd. Chromatograms were analyzed

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using the Chromas software (Technelysium). Sequence data were deposited in GenBank and the accession No. was AB085816.

ORF prediction and promoter analysis

To annotate the sequence, putative open-reading frames (ORFs) were identified with ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Initial functional assignments and homology identifications were made by comparison of the translated ORFs to proteins in the BLAST database with BlastP (http://www.ncbi.nlm.nih.gov/BLAST/). Predicted operons, promoters, and terminators were identified with the tools at Softberry (http://www.softberry.com/berry.phtml). ORF nucleotide coding sequence and deduced amino acid sequence were aligned by ClustalW with default parameters.

Protein expression and functional analysis

The target fragments were ligated to the same restriction sites as of pBV220 vector to obtain pBV220-*phbA* or pBV220-*phbB* or pBV220-*phbC* by standard procedures. The recombinant plasmids were used to transform into *E. coli* TG1 competent cells using the heat shock method. The recombinants were confirmed by restriction enzyme digestion, agarose gel electrophoresis and PCR. *E. coli* cells were harvested at 4 h following transfer of the culture plates from 37° to 42°C. Cultures maintained at 30°C were harvested in parallel as a control. Electrophoresis on a 12.5% SDS polyacrylamide gel was normally used to analyze the recombinant protein. SDS-PAGE was performed in the Mini-Protean system. After electrophoresis, the gels were stained with Coomassie brilliant blue R250 to visualize the protein bands.

Enzyme activity measurement

E. coli cells were suspended in 50 mM phosphate buffer, pH 7.0, and then disrupted by ultrasonication at 4°C. The activities of β-ketothiolase and acetoacetyl-CoA reductase were assayed according to the method of Oeding and Schlegel (1973). PHB synthase activity was determined according to the modified method of Haywood et al. (1991). Enzyme activity was based on measurements of the product concentrations: CoA and NADPH at 340 nm and CoA at 412 nm.

RESULTS

PHB sequence analysis and operon structure

The entire PHB operon of BJ-1 was sequenced in both strands, and its linkage map is shown in Figure 1. The sequence analysis of the PHB operon (9110 bp) revealed five putative ORFs (Figure 1). Nucleotide sequence analysis revealed that the genes encoding *phbA*, *phbB* and *phbC* are separated by ~80 bp. This short distance between the genes, which have the same transcription direction, without any promoter region, suggests that *phbA*, *phbB* and *phbC* are co-transcribed. The first ORF (*phbC*) encoded a 608-amino acid protein, where the entire sequence showed significant sequence identity to known PHB synthesis genes, with 73% sequence identity with the amidase of the PHA polymerase gene of *Burkholderia* sp

DSMZ 9242 strain (Figure 2). The amino acid sequence alignment showed that the N-terminal of the protein is oppositely conserved, where only two Gln are not identical to amidase of the PHA polymerase gene.

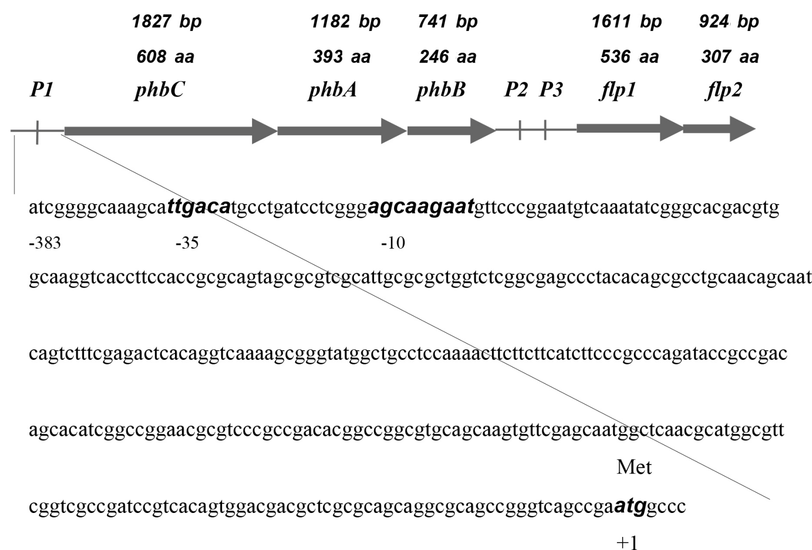

Figure 1. Genetic organization of the BJ-1 PHB operon and adjacent region. The number of amino acids and molecular mass are indicated. Sequence from *phbC* upstream region is shown. Hypothetical -10 and -35 boxes for P1 promoter, and the start codon of *phbC* are indicated.

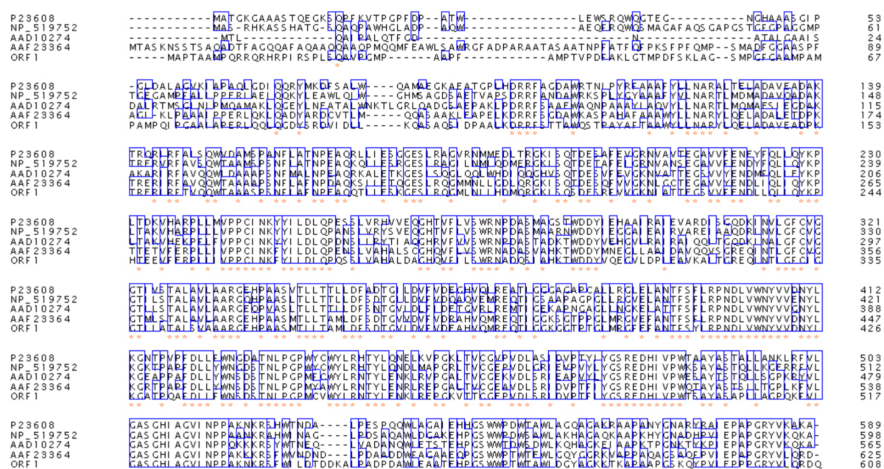

Figure 2. Multiple alignment analysis of open-reading frame 1 (ORF1) and four other protein sequences. AAF23364: *phbC* in *Burkholderia* sp DSMZ 9242, 73% identity to ORF1; P23608: *phbC* in *Ralstonia eutropha*, 63% identity to ORF1; NP_519752: *phbC* in *R. solanacearum*, 61% identity to ORF1; AAD10274: *phbC* in *Alcaligenes latus*, 57% identity to ORF1; ORF1: in this study.

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ORF2 (*phbA*) encoded a 283-amino acid protein, with 89% sequence identity with acetyl-CoA acetyltransferase of *Burkholderia* sp DSMZ 9242 (Figure 3). ORF3 (*phbC*) encoded a 183-amino acid protein, where BLAST searches indicated that it had significant similarities to acetoacetyl-CoA reductase of many bacteria, showing that ORF3 is the gene of synthesizing enzyme NADPH-dependent acetoacetyl-CoA reductase in *Pseudomonas* sp BJ-1 (Figure 4). However, ORF4 encoded a 371-amino acid protein, where BLAST searches indicated similarities to MiaB-like tRNA modifying enzyme protein with 91% sequence identity. The ORF5 gene encoded a 225-amino acid protein, where BLAST searches indicated sequence with high identity to energy metabolism genes in *Pseudomonas*.

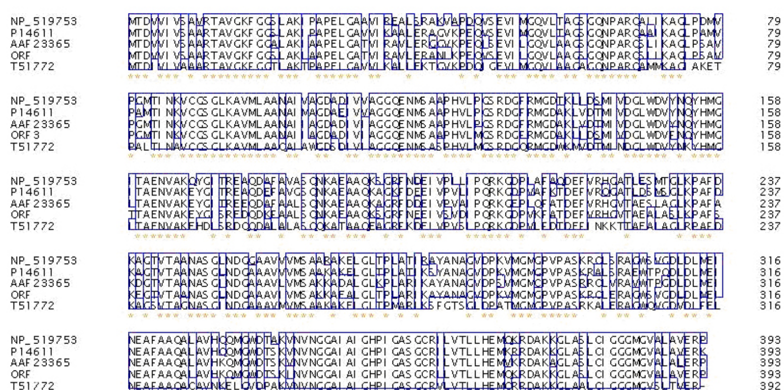

Figure 3. Multiple alignment analysis of open-reading frame 2 (ORF2) and four other protein sequences. NP_519753: *phbA* in *Ralstonia solanacearum*, 86% identity to ORF2; P14611: *phbA* in *R. eutropha*, 84% identity to ORF2; AAF23365: *phbA* in *Burkholderia* sp DSMZ 9242, 89% identity to ORF2; T51772: *phbA* in *Alcaligenes latus*, 84% identity to ORF2; ORF2: in this study.

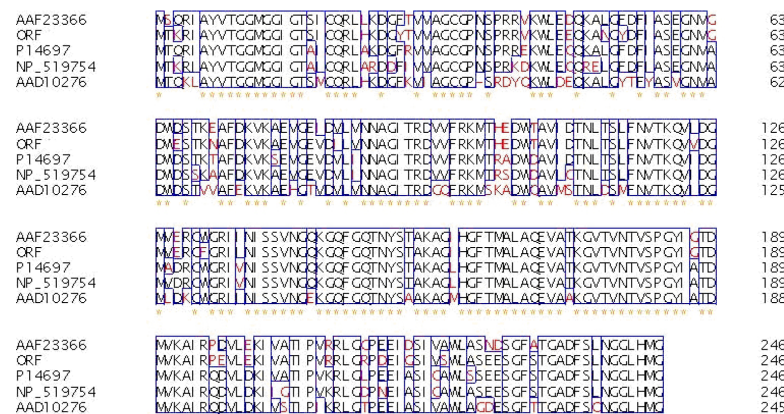

Figure 4. Multiple alignment analysis of open-reading frame 3 (ORF3) and four other protein sequences. AAF23366: *Burkholderia* sp DSMZ 9242, 91% identity to ORF3; P14697: *Ralstonia eutropha*, 85% identity to ORF3; NP_519754*: R. solanacearum*, 83% identity to ORF3; AAD10276: *Alcaligenes latus*, 75% identity to ORF3; ORF3: in this study.

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Promoter analysis

The promoter prediction used the promoter database and the Softberry Plant Regulatory motifs database. We named the promotors Pro1, Pro2 and Pro3 (Figure 1). Pro1 controls the expression of ORF1, ORF2 and ORF3, and Pro2 and Pro3 control the expression of ORF4 and ORF5. One of these 5' mRNA termini is located in front of the *phbC* gene, and the two promoters are located in front of the *flp1* gene. Some other sequences resembling -35 regions TTGACA and -10 regions AGCAAGAAT, are present upstream of the *phbC* gene; the biological meaning of these regions needs further studies.

Functional analysis of PHB operon

To explore whether *phb* gene expression would result in the synthesis of PHB, three expression vector constructs were used in the investigation of recombinant protein production in *E. coli* TG1. The cloned genes were expressed from the tightly regulated temperature-inducible P_RP_L promoter of pBV220. The results are shown in Figure 5: PHBA, PHBB and PHBC were all expressed at high levels as soluble protein. PHBA showed an enhanced expression of a 42-kDa protein when adapted to growth at a temperature from 30° to 42°C. PHBB showed an enhanced expression of a 26-kDa protein and PHBB expression of a 63-kDa protein when adapted to growth at a temperature from 30° to 42°C. The expression of the three proteins confirmed that vector expression was controlled by high temperature.

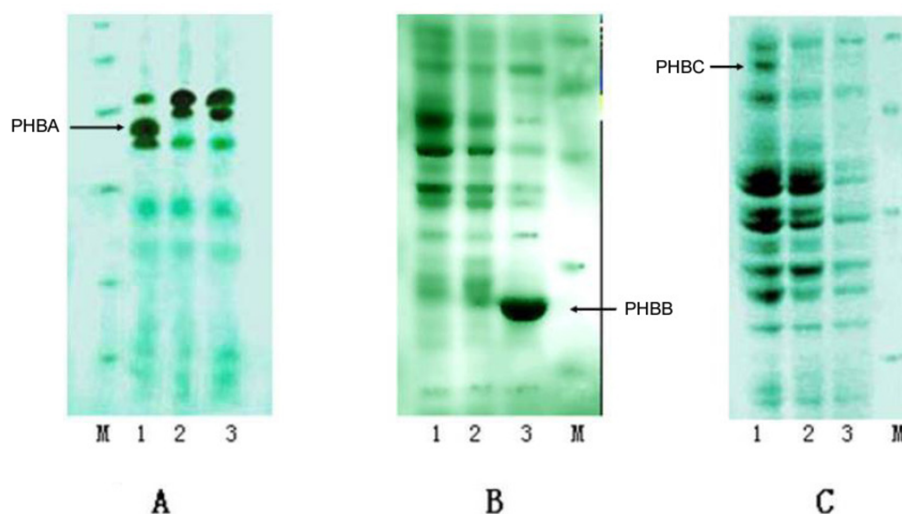

Figure 5. SDS-PAGE analysis of expression of *phbA*, *phbB* and *phbC*. **A.** *phbA* expression analysis. *Lane 1*: the expression of *phbA* induced by 42°C; *lane 2*: the expression of *phbA* induced by 30°C; *lane 3*: the expression of pBV220 induced by 42°C; M: protein molecular marker. **B.** *phbB* expression analysis. *Lane 1*: the expression of pBV220 induced by 42°C; *lane 2*: the expression of *phbB* induced by 30°C; *lane 3*: the expression of *phbB* induced by 42°C; M: protein molecular marker. **C.** *phbC* expression analysis. *Lane 1*: the expression of *phbC* induced by 42°C; *lane 2*: the expression of pBV220 induced by 42°C; *lane 3*: the expression of *phbC* induced by 30°C; M: protein molecular marker.

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The effect of the transformed *phbA*, *phbB* and *phbC* genes on PHB biosynthesis in the *E. coli* recombinants was followed by measuring the intrinsic activity of enzymes involved in the PHB biosynthesis pathway. To investigate the effect of the addition of substrate to PHB enzymes, a series of expression experiments were performed with substrate. As shown in Table 1, the activities of three enzymes β-ketothiolase, acetoacetyl-CoA reductase and PHB synthase were 0.69, 0.58, 0.76 units/mg, respectively. The effective enzyme activity showed that the three genes had biological function in the PHB synthesizing approach.

Table 1. Effect of PHB genes on enzyme activities related to PHB biosynthesis.

Vector	Enzyme activities (units/mg protein)		
	phbA	*phbB*	*phbC*
pBV220-*phbA*	0.69 ± 0.02	-	-
pBV220-*phbB*	-	0.58 ± 0.01	-
pBV220-*phbC*	-	-	0.76 ± 0.02

- = not tested.

DISCUSSION

The production of PHA by different bacteria has drawn attention in recent times because of their use as biodegradable plastic (Liu et al., 2007). There have recently been reports that PHB genes have high sequence difference in different strains (Peralta-Gil et al., 2002). In the present study, we report on the identification and molecular characterization of *phbA*, *phbB* and *phbC*, three genes whose nucleic acid sequence contains some single-nucleotide polymorphisms and indels compared to formerly cloned genes. This may be essential for both PHB accumulation and PHA synthase activity in *Pseudomonas* sp BJ-1.

The three genes are related to PHB biosynthesis by bioinformatics prediction. The operon related to the genes for PHB biosynthesis from *Pseudomonas* sp BJ-1 was analyzed by protein expression. Many special bacteria have a similar synthesis approach, where five genes are involved in PHB biosynthesis in *Alcaligenes eutrophus* (Steinbuchel and Schlegel, 1991). We predicted five ORFs by gene prediction software, and protein function was deduced by sequence similarity. ORF1, ORF2 and ORF3 are similar to PHB biosynthesis genes, and ORF1 and ORF2 are conserved with respect to known genes. The three ORF are controlled by the same promoter, confirming that the three genes have identical function. The ORF4 and ORF5 are controlled by Pro4 and Pro5, where the two gene functions are not clear but similar to that of the MiaB-like tRNA modifying enzyme family (Anton et al., 2008). Although the genes are not directly involved with PHB biosynthesis, we presume that the genes are linked to energy metabolism in PHB biosynthesis.

The PHB biosynthesis operon structure and sequence are similar to published operons of other *Pseudomonas*, but these genes encode a different protein sequence (Takeda et al., 2000). There are two possible reasons for this phenomenon. One is that PHB biosynthesis genes show diversity in different bacteria. Another is that there are several types of PHB biosynthesis operons in bacteria. Different operon genes encode different isozymes, and thus, sequence variation is apparent among these genes.

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